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Analysis of Three Variables in Sampling Solutions Used to Assay Bacteria of Hands: Type of Solution, Use of Antiseptic Neutralizers, and Solution Temperature

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Tests were performed using the sterile bag technique to determine the effects of type of sampling solution, use of antiseptic neutralizers, and solution temperature on the detection and quantitation of bacteria on hands. Using paired hand cultures, three sampling solutions were compared: quarter-strength Ringer solution, a phosphate buffer containing Triton X-100, and the same buffer containing antiseptic neutralizers. The phosphate buffer containing Triton X-100 was significantly better than quarter-strength Ringer solution in mean bacterial yield; the neutralizer-containing sampling solution was slightly better than Triton X-100-containing solution, although differences were not significant at the $P = 0.05$ level. Temperature (6 or 23°C) of the sampling solution showed no consistent effect on bacterial yield from hands tested with the fluid containing neutralizers.

Culturing bacteria of hands has been used in health care institutions since the 1930s during investigations to search for reservoirs of epidemic-causing organisms and as a surveillance technique to evaluate general levels of cleanliness in the environment. Carriage of gram-negative bacilli on hands of hospital personnel has often been implicated as a mode of spread within hospitals (10, 11, 22). There are numerous techniques for assaying the bacterial flora of hands. The three most commonly used are the contact or impression plate, the swab, and the gloved hand or sterile bag techniques. Each has slightly different usefulness; the advantages and disadvantages of each are summarized in Table 1.

The technically simple contact plate method and the more sensitive swab method permit sampling of large numbers of subjects with little disturbance even in a busy hospital environment. The gloved hand or bag method is more cumbersome and time-consuming but much more suitable for sampling the complete hand, even though the unavoidable dilution reduces sensitivity.

We were interested in detection, identification, and quantitation of gram-negative organisms with potential significance in nosocomial infections and quantitation of other organisms identified only as to major groups, such as the family *Micrococcaceae*. After preliminary trials with the contact culture method, we realized that small numbers of our target organisms were readily missed, and we shifted to the sterile bag technique, a modification of the serial basin technique of Price (18). This culture method has been adapted by others (22). A wide variety of sampling solutions have been used to assay bacteria of the skin, including sterile water (10, 22), sterile saline (15), nutrient broth (3), Ringer solution (2, 5, 13), and a surface-active agent buffer solution (14, 23, 25).

These sampling solutions were used with and without the addition of antiseptic neutralizers. Neutralizers in the sampling solution or culture media or both have been reported by some investigators to neutralize residual antiseptic and to improve bacterial yield (6, 7, 20). M. W. Casewell, on the other hand, reported equally good recovery of *Klebsiella* from sampling solutions with and without the added neutralizers (M.D. thesis, University of London, London, England, 1977). Principal neutralizers used in past studies include Tween 80 to neutralize phenolics (7, 12); lecithin to neutralize phenolics, quaternary ammonium compounds, and chlorhexidine (6, 13); and sodium thiosulfate to neutralize iodine and chlorine (6). Types and concentrations of neutralizers used in published studies of hand flora are listed in Table 2.

Thus, there are two technical variations of the sterile bag or gloved hand culture technique which have unknown influences on bacterial yield: the choice of sampling solution and the use of antiseptic neutralizers in the solution or culture media. This report describes tests of the sterile bag technique to determine (i) whether there is a difference in bacterial yield obtained when two different sampling solutions are used

TABLE 1. *Frequently used techniques for culturing hands*

Technique	Representative studies	Purpose	Advantages	Disadvantages
Contact (impression) plate	Gale et al. (9), Berman and Knight (4), Kominos et al. (11), Ojajärvi et al. (16)	Screening for gross levels of hand contamination, especially during hospital outbreaks	Least expensive, technically simple	Not quantitative, very insensitive; colonies may be very close, difficult to count and identify
Swab	Evans and Stevens (8), McBride et al. (14), Aly and Maibach (1), Shaw et al. (23)	Quantitative analysis of small areas of skin	Permits satisfactory quantitation and identification of surface and a variable proportion of subsurface organisms	Permits sampling of only small areas at one time; cannot readily estimate bacterial population of a larger surface area, such as entire hand
Gloved hand or sterile bag	Salzman et al. (22), Lowbury and Lilly (13),[a] Michaud et al. (15), Knittle et al. (10), Casewell and Philips (5)	Quantitation of flora of total hand surface	As for the swab technique, but for a larger surface area	More complicated procedure; initial dilution in sampling fluid reduces sensitivity

[a] Fingers are sampled in a tube containing glass beads.

TABLE 2. *Antiseptic neutralizer combinations used in published studies of hand flora*

Investigator	Neutralizer concn	Neutralizer used in:	
		Sampling solution	Culture media
Lowbury and Lilly (13)	1% Lubrol[a]-0.5% lecithin	×	×
Shaw et al. (23)	1% Tween 80		×
Michaud et al. (15)	"Letheen" agar (0.5% Tween 80-0.07% lecithin)		×
Petersen et al. (17)	For povidone-iodine-washed hands: 0.02% sodium thiosulfate	×	
	For hexachlorophene-washed hands: 0.02% Tween 80	×	
Knittle et al. (10)	0.5% Tween 80-1% sodium thiosulfate-0.07% lecithin		×[b]
McBride et al. (14)	0.1% Tween 80		×
Aly and Maibach (1)	10% Tween 80-3% lecithin	×	
Ojajärvi et al. (16)	1% Tween 80		×
Ayliffe et al. (3)	0.75% Tween 80-lecithin mix-1% sodium thiosulfate	×	

[a] A British proprietary product recommended as a neutralizer of chlorhexidine (13). Not available in the United States.

[b] Was found to have no effect on bacterial recovery and was subsequently omitted.

(quarter-strength Ringer solution or a phosphate buffer, pH 7.9, containing 0.1% Triton X-100 originally described by Williamson and Kligman [25]), (ii) whether the addition of antiseptic neutralizers to the sampling solution alters the bacterial yield, and (iii) whether there is a difference between the bacterial yield obtained when the sampling solution is cold (6°C) and that obtained at room temperature (about 23°C).

MATERIALS AND METHODS

Culturing technique. For the first two tests, large sterile gloves were used for sampling hand bacteria. However, we found the gloves difficult to remove. In subsequent experiments, therefore, 1-quart (ca. 0.95-liter) polyethylene bags (Ziploc storage bags; Dow Chemical Co., Indianapolis, Ind.), sterilized with ethylene oxide, were substituted and proved satisfactory. Tests performed on both the bags and the gloves showed them to be sterile and not inhibitory to bacterial growth.

Fifty milliliters of sterile sampling solution was poured into the bag, and, after inserting the hand, the subject held the bag opening around the wrist. The fingers, particularly around the nails, and the palm, but not the back of the hand, were rubbed through the wall of the bag by the investigator for 1 min. The solution was then either poured into a sterile container and agitated up and down 25 times in 7 s according to the American Public Health Association standard plate count method for the quantitation of bacteria in water (21) or was mixed in the bag, from which aliquots of solution were removed directly. The variation in bacterial recovery from the right and left hands of an individual is random and should not introduce bias (15, 19). In these tests we used the hands of each individual as a paired sample.

Comparison of sampling solutions. We selected two solutions to test. We chose quarter-strength Ringer solution (NaCl, 2.25 g; KCl, 0.075 g; $CaCl_2 \cdot 2H_2O$, 0.0625 g; $NaHCO_3$, 0.05 g; distilled water, 1 liter) because it has been used in a number of studies, including one recently published major study of gram-negative bacilli on the hands (5), and because it

seemed probable that the numbers of bacteria recovered with some other sampling solutions, such as plain water or physiological saline, without a surface-active agent, would be no greater than those recovered with the dilute Ringer solution. The other solution tested, a 0.075 M phosphate buffer, pH 7.9, with 0.1% Triton X-100 (New England Nuclear Corp., Boston, Mass.), henceforth referred to as TriBuf, has lipid dispersal qualities shown to be effective in maximizing bacterial yield from the skin (25).

Subjects for the tests were faculty or staff of the University of Washington Schools of Medicine and Nursing, Seattle, Wash. None were involved with clinical or laboratory work that resulted in unusually frequent handwashing. Each refrained from handwashing for at least 20 min, and in most cases several hours, before culture. Subjects routinely used bar soap rather than antiseptic agents for handwashing and bathing.

Three paired cultures were performed on each of five subjects, for a total of 15 tests. In each of the three cultures which were at least 24 hours apart, one hand was sampled with TriBuf solution and the other was sampled with quarter-strength Ringer solution.

Portions (0.05 ml) of each sampling solution were plated on our standard agar medium containing 3% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), 1.0% yeast extract, and 0.1% Tween 80 (polyoxyethylene sorbitan monooleate; Sigma Chemical Company, St. Louis, Mo.) (TSY-TW). Inoculum was spread with bent glass rods. When neutralizers were added to the sampling solution, the same medium without Tween 80 was used. The plates were incubated aerobically for 24 h at 37°C. Total colony counts were made, and Gram stains were performed on each colony type. When gram-negative bacteria were encountered, they were subcultured and identified by a commercially available multitube system (Enterotube II; Roche Diagnostics, Nutley, N.J.).

Effect of addition of neutralizers to sampling solution. Since iodophors, chlorhexidine, and hexachlorophene preparations are commonly used by personnel in health care institutions, neutralizers for all three antiseptic types were included in the tested sampling solution. Proportions of each were chosen based on reported studies (6) and communications with microbiologists experienced in this aspect of culturing organisms from the skin (F. B. Engley, University of Missouri, Columbia, Mo.; W. Sheikh, Stuart Pharmaceuticals, Wilmington, Del.). The basic diluent was the same phosphate buffer with Triton X-100 omitted. Neutralizers added were 0.5% lecithin (Asolectin; Associated Concentrates, Long Island, N.Y.), 0.5% sodium thiosulfate, and 1.0% Tween 80. Neutralizers were used in the sampling solution, but not in the media.

Potential toxicity of the neutralizer-containing sampling solution (henceforth referred to as NB) was tested with five species: *Escherichia coli*, *Acinetobacter calcoaceticus* subsp. *anitratus*, *Staphylococcus epidermidis*, *Enterobacter aerogenes*, and *Serratia marcescens*. Bacteria were obtained from human skin or from stock supplies of the Department of Microbiology and Immunology, University of Washington. Each of the species, in quantities of 10^4 to 10^6 colony-forming units (CFU), was inoculated into a tube containing 2 ml of NB and a tube containing 2 ml of TriBuf. Sequential 10-fold dilutions of each species in each test solution were made. At 0, 5, 15, and 60 min, 0.05-ml portions of the NB and TriBuf solutions containing each test species were plated on MacConkey agar (Difco Laboratories, Detroit, Mich.) or TSY-TW agar in petri dishes (100 by 15 mm) with two compartments (biplates). Plates were incubated aerobically for 24 h at 37°C, and colonies were counted.

The two solutions were compared in cultures of hand flora of four subjects. First, two subjects had paired unwashed-hand samples taken with the test solutions, a total of four tests. Next, four subjects had 3 paired samples taken (12 samples total) after hand washing with a 4.0% (wt/vol) chlorhexidine gluconate solution (Hibiclens; Stuart Pharmaceuticals, Wilmington, Del.). The hands were washed with the chlorhexidine-containing cleaner according to the manufacturer's directions for health care personnel (wet hands, place about 5 ml of antiseptic in one palm, wash vigorously for 15 s, rinse, and dry). After a 5-min wait, during which time "clean" activities were carried out (reading or writing, for example), the hands were cultured, one hand with NB and the other with TriBuf. As with all other tests, solutions were used on the opposite hand, with subsequent tests. Cultures were repeated no less than 24 h apart and in most cases were more than 72 h apart.

Effect of sampling solution temperature on bacterial yield. In one of our tests we noted greater than usual differences in bacterial yield between a subject's hands when one hand was tested with cold solution and the other was tested with solution at room temperature. To determine whether this was a chance occurrence, a series of 15 tests on three subjects was performed, 6 tests for each of two subjects and 3 tests for the other subject. Hands were washed with chlorhexidine-containing solution in the usual manner. We were especially concerned about survival of gram-negative organisms at the two temperatures. Therefore, 5×10^5 to 5×10^7 CFU of *E. coli* in 0.05 ml were inoculated onto the hypothenar eminence. The inoculum was spread for 10 s with a glass rod across the upper palm. After 3 min of drying, the hand was sampled in the usual manner with NB at room temperature (23°C). The other hand was likewise inoculated and cultured with cold (6°C) NB. MacConkey agar biplates were inoculated with 0.05-ml amounts of the sampling solutions and incubated for 24 h at 37°C, and total *E. coli* colonies were counted. To determine the number of CFU deposited on the hands, the inoculum of *E. coli* was titrated in each test by serial dilutions plated on MacConkey agar.

RESULTS

Comparison of sampling solutions. The organisms found in the tests of the two solutions were almost all gram-positive cocci with the microscopic appearance and colonial morphology commonly associated with *Micrococcaceae*. The numbers of CFU recovered ranged from 4.0

$\times 10^2$ to 2.1×10^4/ml of sampling solution (Table 3).

In 14 of 15 tests TriBuf yielded higher mean colony counts than did dilute Ringer solution. The ratio of CFU from TriBuf to CFU from Ringer solution ranged from 8.8:1 to 1:1.3, with a median value of 1.9:1. A test of the null hypothesis that both solutions would have equal ($P = 0.5$) probabilities of yielding higher mean counts was significant at $P < 0.01$. A paired t-test of the null hypothesis that there was no difference in mean colony counts between the two solutions was also significant at $P < 0.001$. Thus, we concluded that TriBuf was the superior sampling solution.

TABLE 3. *CFU recovered from the hands of five subjects in paired tests of quarter-strength Ringer solution and TriBuf*

Subject (sex, age)	CFU/ml of sampling solution		TriBuf/ Ringer ratio
	TriBuf	Ringer	
F, 36	3.5×10^3	4.0×10^2	8.8:1
	2.1×10^4	1.1×10^4	1.9:1
	1.1×10^4	1.7×10^3	6.5:1
M, 37	1.8×10^4	1.5×10^4	1.2:1
	8.3×10^3	7.1×10^3	1.2:1
	7.3×10^3	5.4×10^3	1.4:1
M, 25	4.4×10^3	1.6×10^3	2.8:1
	1.9×10^4	1.5×10^4	1.3:1
	7.0×10^3	9.2×10^2	7.6:1
M, 67	1.3×10^4	5.8×10^3	2.2:1
	1.4×10^4	7.7×10^3	1.8:1
	2.0×10^4	2.8×10^3	7.1:1
F, 30	2.7×10^3	3.6×10^3	1:1.3
	1.3×10^3	4.2×10^2	3.1:1
	9.4×10^3	7.4×10^2	1.3:1

TABLE 4. *CFU recovered from the hands of four subjects, using NB and TriBuf*

Subject (sex, age)	CFU/ml of sampling solution		NB/ TriBuf ratio
	NB	TriBuf	
Unwashed hands			
F, 36	1.9×10^4	1.5×10^4	1.3:1
	2.8×10^4	2.0×10^4	1.4:1
M, 37	3.0×10^4	3.6×10^4	1:1.2
	3.0×10^4	1.5×10^4	2.0:1
Washed hands			
F, 36	1.4×10^4	7.0×10^3	2.0:1
	5.4×10^3	4.2×10^3	1.3:1
	8.4×10^3	7.2×10^3	1.2:1
M, 37	2.2×10^4	4.2×10^3	5.2:1
	5.9×10^4	2.3×10^4	2.6:1
	2.6×10^4	1.9×10^4	1.4:1
M, 25	2.0×10^4	1.6×10^4	1.2:1
	1.9×10^4	2.5×10^4	1:1.3
	7.6×10^3	5.4×10^3	1.4:1
F, 25	3.2×10^3	1.5×10^3	2.1:1
	2.6×10^3	1.7×10^3	1.5:1
	1.0×10^3	1.3×10^3	1:1.3

TABLE 5. *CFU of artificially inoculated E. coli recovered from the hands of three subjects, using sampling solution at 6°C and at about 23°C*

Subject (sex, age)	CFU/ml of sampling solution at:		6°C/23°C ratio
	6°C	23°C	
F, 36	5.2×10^4	1.6×10^4	3.2:1
	8.8×10^3	1.8×10^4	1:2.0
	3.1×10^4	1.0×10^3	31.0:1
	2.0×10^3	6.9×10^3	1:3.4
	5.6×10^2	9.0×10^2	1:1.6
	1.9×10^2	2.4×10^3	1:12.6
M, 25	8.9×10^3	1.9×10^4	1:2.1
	2.2×10^3	4.0×10^2	5.5:1
	2.9×10^4	5.4×10^3	5.4:1
	6.0×10^2	3.0×10^2	2.0:1
	8.0×10^2	1.9×10^3	1:2.4
	3.0×10^1	6.0×10^3	1:200
M, 67	2.9×10^5	4.2×10^3	69.0:1
	4.4×10^3	1.8×10^3	2.4:1
	3.0×10^4	2.2×10^4	1.4:1

Effect of addition of neutralizers to sampling solution. All organisms retrieved from washed hands were gram-positive cocci and coryneform organisms. Proportions of organism types varied slightly between sampling solutions, but this was probably a random phenomenon. There was, however, an unexpected finding with the unwashed hands of one subject. On two successive cultures, taken 2 days apart on opposite hands, *A. calcoaceticus* was recovered from the hand sampled with NB, but not on the TriBuf-sampled hand. In one sample *A. calcoaceticus* accounted for 17% of the total flora (3.2×10^4 CFU/ml); in the other sample it accounted for 0.3% (85 CFU/ml). These were the only instances of retrieval of gram-negative bacteria in the 16 tests.

For both washed and unwashed hands, the range of CFU was 1.0×10^3 to 5.9×10^4/ml of sampling solution. Of the 16 tests, 13 yielded higher mean colony counts with NB and 3 yielded higher mean counts with TriBuf (Table 4). A test of the null hypothesis that the two solutions would have equal ($P = 0.5$) probabili-

ties of yielding higher mean colony counts was significant at $P = 0.08$. A paired t-test of the null hypothesis that there was no difference in mean colony counts between the two solutions resulted in $0.1 < P < 0.05$. Thus, the NB solution had a greater proportion of tests with a higher yield, but the results were not significant at $P = 0.05$. The median ratio of CFU recovered with NB as compared with CFU recovered with TriBuf was 1.35:1 for unwashed hands and 1.4:1 for hands washed with the chlorhexidine-containing cleanser.

In tests of the stability of four species of gram-negative rods and *S. epidermidis* in sampling solutions, there was no decrease in the five species after 60 min in either NB or TriBuf. In two tests *E. coli* showed an 85 to 95% drop in CFU after 60 min in TriBuf, but no drop in NB. There was no measurable decrease in *E. coli* in either solution at 5 min. Thus, there was no killing of these bacteria by the neutralizers in the proportions used.

Effect of sampling solution temperature on bacterial yield. There was a broad range of bacterial recovery after inoculation of *E. coli* on the hands. Numbers of CFU per ml of sampling solution ranged from 3.0×10^1 to 2.9×10^5 for cold solution and from 4.0×10^2 to 2.2×10^4 for room temperature solution. The ratios of cold (6°C) bacterial recovery to room temperature (23°C) recovery are displayed in Table 5. In 8 of 15 tests, bacterial counts were greater for cold than for room temperature solution; in 7 of 15 tests, counts were greater for room temperature than for cold solution. This was clearly not different from chance. Therefore, there was no consistent effect on bacterial yield from the temperature of the sampling solution.

DISCUSSION

In the lipid-rich substrate on human skin bacteria clump, and colony counts will underestimate the microbial population unless organisms are dispersed (24). Triton X-100 and Tween 80 disperse these clumps so that more organisms become countable. This is probably the principal reason that TriBuf solution was clearly superior to quarter-strength Ringer solution for hand culturing.

Higher mean colony counts were obtained with NB than with TriBuf in 75% of the tests in which they were compared. Higher counts with NB as compared with TriBuf might have resulted by chance or might have been due to the dispersing qualities of Tween 80 or to a better yield of bacteria after residual antiseptic on the hands was neutralized. Regardless of the mechanism involved, it is clear that adding neutralizers did not reduce the numbers of bacteria recovered. Since those whose hand flora are of interest, such as hospital personnel, are likely to use antiseptics containing iodine, hexachlorophene, or chlorhexidine, use of neutralizers in sampling solutions is indicated.

The sampling solution with neutralizers for antiseptics provided the necessary dispersion of skin flora and, as far as our tests indicated, had no disadvantages. The fact that *A. calcoaceticus* was isolated from the unwashed hands of one subject on two separate occasions with the NB solution, but not with the TriBuf solution, raises the question of whether it is superior for the recovery of certain organisms, a question worthy of further study.

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